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Dynamic expression patterns of progenitor and neuron layer markers in the developing human dentate gyrus and fimbria

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Running title:

Early development of the human dentate gyrus

Abstract

The molecular mechanisms that orchestrate the development of the human dentate gyrus are not known. In this study, we characterized the formation of human dentate and fimbrial progenitors and postmitotic neurons from 9 gestational weeks (GW9) to GW25. $PAX6^+$ progenitor cells remained proliferative until GW16 in the dentate VZ. By GW11, the secondary dentate matrix had developed in the intermediate zone, surrounding the dentate anlage and streaming towards the subpial layer. This secondary matrix contained proliferating $PAX6^+$ and/or $TBR2^+$ progenitors. In parallel, $SOX2^+$ and $PAX6^+$ fimbrial cells were detected approaching the dentate anlage, representing a possible source of extra-dentate progenitors. By GW16, when the granule cell layer could be delineated, a hilar matrix containing $PAX6^+$ and some $TBR2^+$ progenitors had become identifiable. By GW25, when the two limbs of the granule cell layer had formed, the secondary dentate matrix was reduced to a pool of progenitors at the fimbrio-dentate junction. Although human dentate development recapitulates key steps previously described in rodents, differences seemed to emerge in neuron layer markers expression. Further studies are necessary to better elucidate their role in dentate formation and connectivity.

Keywords: fimbria, granule cell neurogenesis, progenitors, dentate matrix, neuron layer markers

Introduction

The hippocampal formation consists of the hippocampus proper with subfields CA3-CA1, the dentate gyrus (DG) and the subiculum. Together with the adjacent entorhinal cortex (EC), it is involved in encoding detailed information about novel events into long-term memory (for reviews, see refs (Amaral et al., 2007). In a recent work, we have characterized the development of progenitors and pyramidal neurons of the hippocampal formation and EC, providing evidence for the lamination of the pyramidal layer (Cipriani et al., 2015).

The DG is a simple structure characterized by unidirectional excitatory microcircuitry, and composed of the molecular layer, the granule cell layer and the polymorphic layer (also called the hilus), which contains mossy cells and GABAergic interneurons. The DG receives major projection carrying sensory information of multiple modalities from the EC, via the perforant path (Amaral and Kurz, 1985; Amaral et al., 2007; Witter, 2007). The DG also receives minor projections from deep layers of the EC (for review, see (Witter, 2007). DG projects to hilar mossy cells and CA3 pyramidal neurons (Blaabjerg and Zimmer, 2007).

Studies of hippocampal ontogenesis suggest that substantial differences might exist between primates and rodents (Seress and Mrzljak, 1992; Insausti, 1993; El-Falougy and Benuska, 2006; Scharfman, 2011). In fact, in contrast to other mammals, the hippocampus of primates develops adjacent to the ventral cortical hem and is therefore medioventrally located (West, 1990; Abraham et al., 2004).

The main steps of the formation of the DG have been largely studied in rodents (Altman and Bayer, 1990a, c, b; Frotscher et al., 2007). However, the molecular programs that control neuronal subtype specification in the DG have not been well characterized (Simon et al., 2012; Nielsen et al., 2014). The generation and migration of mammalian dentate neurons are complex processes (Rakic and Nowakowski, 1981; Altman and Bayer, 1990c) originating in different germinal compartments (Altman and Bayer, 1990a, b). As in the pyramidal layer, the first progenitors of the DG are generated by the dentate ventricular zone (VZ). A second germinal zone, called the secondary dentate matrix, forms later in the intermediate zone, and

extends tangentially towards the dentate anlage, leading to the so-called “dentate matrix migration” (Altman and Bayer, 1990a). In addition, this secondary matrix also establishes a proliferating area within the hilus that in turn gives rise to the neurogenic subgranular zone in adult life (Altman and Bayer, 1990a, b; Nakahira and Yuasa, 2005; Eriksson et al., 1998; Seri et al., 2001; Lee et al., 2002; ;Knoth et al., 2010; Akers et al., 2014). However, only a few studies have examined the molecular mechanisms that regulate dentate neurogenesis during development (Abraham et al., 2004; Gu et al., 2011; Hodge et al., 2012; Hodge et al., 2013; Roybon et al., 2009). In mice, Tbr2 is required for the development of intermediary precursor cells and of the granule cell layer (Hodge et al., 2012). In addition, the transcription factor Ctip2 has been reported to regulate granule cell formation in mice (Simon et al., 2012). However, the pattern of expression and the function of molecular layer markers have not been well investigated during the development of the DG, and it is not clear whether other layer-specific transcription factors could also play a role.

The aim of the present study was to examine the fundamental processes of the development of the human DG, focusing on the germinal areas and the hem/fimbria, which has been shown to be a source of Cajal-Retzius cells in mice and humans (Abraham et al., 2004; Gu et al., 2011; Hodge et al., 2013), and the expression of transcription factors that regulate neuronal subtype specification and layering in the neocortex.

Materials and Methods

Human tissue procurement and processing

Fifteen human fetuses aged between 9 and 25 gestational weeks (GW, or post-ovulatory week) without any neuropathological alterations were collected after legal abortion or spontaneous death (Table 1). All procedures were approved by the ethics committee (Agence de Biomédicine; approval number: PFS12-0011). Tissues were immersion-fixed in 4% paraformaldehyde and/or formalin. For frozen sections, samples were cryoprotected in 20% sucrose and stored at -80 °C until use. Tissues were cut into 12 µm-thick coronal

sections, mounted on Superfrost slides and stored at -80 °C. For paraffin sections, samples were dehydrated through an ethanol gradient, delipidized in xylene, embedded in paraffin and cut into 5 µm coronal sections. Before immunolabeling, antigen retrieval was performed in citrate buffer for 1 hour at 94°C (1.8 mM citric acid, 8.2 mM sodium citrate, pH 6).

Mice tissue

C57BL/6 mice were housed under a 12 h light/dark cycle with food and water ad libitum. Animal procedures were approved by the Debré-Bichat National Ethics Committee (Project No. 2010-13/676-0006) and conformed to French laws on animal protection. Mice were perfused at P5 and P10 with 4% paraformaldehyde. Brains were removed and cryoprotected in 20% sucrose. Samples were stored at -80 °C until use. Tissues were cut into 10 µm-thick sections, mounted on Superfrost slides and stored at -80 °C.

Immunohistochemistry

Frozen and paraffin-embedded sections were permeabilized with 0.1% Triton X-100 dissolved in 0.12 M phosphate buffered saline (PBS-T) for 15 minutes at 25°C. To block non-specific binding, sections were incubated in blocking buffer (10% goat serum in TBS-T, 1 hour at 25°C). Sections were then treated with primary antibodies diluted in blocking buffer (16 hours at 4°C) at the concentrations reported in Table 2. The day after, sections were incubated with Alexa Fluor 488-, Alexa Fluor 565-, or Alexa Fluor 676-conjugated secondary antibodies (1:500, Invitrogen Molecular Probes) diluted in blocking buffer. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, 1 µg/ml, Invitrogen Molecular Probes). Coverslips were mounted using Fluoromount-G mounting medium (SouthernBiotech, Birmingham, USA).

For P73 labeling, immunoreactivity was visualized using the Tyramide Signal Amplification System (PerkinElmer, Waltham, MA) according to the manufacturer's instructions.

For Nissl staining, slides were stained with 0.5% cresyl violet (Sigma; in 0.3 % acid acetic). Sections were differentiated in 70% ethanol and dehydrated in 100% ethanol. After clearing

in xylene, coverslips were mounted using Pertex mounting medium (HistoLab, Gothenburg, Sweden).

Microscopy

Virtual slides in brightfield were created using an Axio Scan.Z1slide scanner (Zeiss, Germany) and processed with Axio Scan.Z1 ZEN (Zeiss, Germany).

Tile scans of fluorescent labeled-sections were acquired using a Zeiss Axio Observer.Z1 fluorescence microscope with the following excitation/emission wavelengths: 359/461 nm for DAPI, 470/509 nm for Alexa Fluor 488, 558/583 nm for Alexa Fluor 555 and 649/670 nm for Alexa Fluor 647, using a Plan-Apochromat 20X/0.8 M27 objective and AxioCamMR3 camera. Images were processed with Axiovision Rel. 4.8 software (Zeiss).

Fluorescent sections were analyzed using a Leica TCS SP8 confocal scanning system (Leica Microsystems) equipped with 405-nm Diode, 488-nm Ar, 561-nm DPSS and 633-nm HeNe lasers. Eight-bit digital images were collected from a single optical plane using a Leica 20x HC PL APO CS2 oil-immersion objective (numerical aperture 0.75) or a Leica 40x HC PL APO CS2 oil-immersion objective (numerical aperture 1.30). Z-stacks were obtained at 0.9 µm intervals with a resolution of 5.285 pixels/micron. Images were processed with LAS AF.Ink software (Leica) and analyzed using ImageJ software (National Institutes of Health, USA). Images were assembled into photomontages using Adobe Photoshop CS2 (Quark Inc., Denver, CA, USA).

For each case, the percentages of the cells were established on individual and merged channels of confocal images taken with a 40x objective. Cells were counted in one field from one optical section of a stack gathered in 1 micrometer steps, using the cell counter plug-in from ImageJ. The density between stacks did not significantly change.

Results

9-10 gestational weeks

In the three cases that we analyzed, the primordium of the hippocampal formation developed next to the hem (Abraham et al., 2004) (Fig. 1*A,B,C*). Proliferative activity and the expression of neuronal-fate-specific transcription factors (or layer markers) were assessed using multiple antibodies and confocal analysis, as previously described for the ammonic formation (Cipriani et al., 2015).

Precursor cells labeled for SOX2 (Fig. 1*D*) and PAX6 (Fig. 1*E*) were observed in the VZ of the primordium of the hippocampal formation. Some of these cells displayed proliferative activity, as shown by colabeling for Ki67 (Fig. 1*D*,*E*). We were not able to differentiate between ammonic and dentate progenitors. A small cluster of proliferating $SOX2^+/Ki67^+$ or $PAX6^+/Ki67^+$ double-labeled progenitors extended from the VZ towards the intermediate zone at the dorsal limit of the hem, likely representing the anlage of the secondary dentate matrix (Fig. 1*D,E*). Some proliferating $PAX6^+/Ki67^+$ progenitors were also observed near the marginal zone (Fig. 1*E*), as previously shown (Cipriani et al., 2015).

11 gestational weeks

In the two cases we collected, the dentate area was identifiable between the hippocampal plate and the hem.

Formation of the dentate anlage and secondary matrix

The putative dentate VZ (the primary matrix) was located between the ammonic VZ and the hem VZ. Although the limits between these compartments were not clearly defined (Fig. 1*F*), $Ki67^+$ cells were less dense in the dentate and hem VZ compared to the ammonic VZ (Fig. 1*F*). In the dentate VZ (primary matrix), $Ki67^+$ cells represented about one third of the all cells

(30±6%), of which some were PAX6$^+$ progenitors (Fig. 1*G*) (Cipriani et al., 2015) and TBR2$^+$ intermediate progenitor cells (Fig. 1*I*)".

At this stage, the secondary dentate matrix could be identified (Fig. 1*F-L*), and formed a C-shape band of proliferating Ki67$^+$ cells, including PAX6$^+$ progenitors (Fig. 1*J*) and TBR2$^+$ intermediate progenitor cells (Fig. 1 *K, L*) surrounding the dentate anlage (Fig. 1 *J-L*). The presence of PAX6$^+$/TBR2$^+$ double-labeled cells suggested the production of TBR2$^+$ intermediate progenitor cells from PAX6$^+$ progenitors (Fig. 1*H*) as previously shown in the cortex and the pyramidal plate of the hippocampus (Cipriani et al., 2015; Haubensak et al., 2004; Noctor et al., 2004; Ochiai et al., 2009). In addition, some PAX6$^+$ and TBR2$^+$ cells were also observed within the dentate anlage, where proliferating cells (Ki67+ cells) represented 8±1% of all cells (DAPI$^+$ cells). A subpopulation of TBR2$^+$ cells, coexpressing NeuroD1$^+$ and delineating the postmitotic TBR2-/NeuroD1$^+$ dentate anlage, partly surrounded the mediodorsal and lateral side of the dentate anlage (Fig. 1*L*, orientation shown in Fig. 1*F*).

This indicates that TBR2$^+$ cells of the secondary dentate matrix were committed to a neuronal fate. In summary, between 9 and 11 GW, the secondary matrix appeared next to the dentate VZ.

We next investigated the expression of layer markers (Alcamo et al., 2008; Britanova et al., 2008; Chen et al., 2008; Cubelos et al., 2008; Bedogni et al., 2010; Cubelos et al., 2010; Han et al., 2011). CTIP2$^+$ cells were mainly present in the core, while CUX1$^+$ neurons predominated in the rim. Some CTIP2$^+$ cells also expressed CUX1 (Fig. 1*M*). TBR1 displayed a reducing gradient from the ammonic (Fig. 1*O*) to the dentate area where the immunolabeling was faint (Fig. 1*N*).

In conclusion, at this stage, the dentate VZ and the secondary matrix represented the two proliferating compartments of the dentate anlage. Putative granule neurons appeared, and

expressed a restricted number of layer markers known to play a role in glutamatergic neurogenesis in the neocortex (Chen et al., 2008; Cubelos et al., 2008; Cubelos et al., 2010).

Progenitor and postmitotic cells of the hem

PAX6$^+$ cells were present in the VZ and in the contiguous intermediate zone, forming a thin tangential band adjacent to the marginal zone (Fig. 1*P*). Some PAX6$^+$ cells were colabeled for TBR2 (Fig. 1*P*). In contrast to the secondary dentate matrix, TBR2$^+$ cells did not display proliferative activity (Fig. 1*Q*) and some of them were colabeled with CUX1 (Fig. 1*R*). As previously described, P73$^+$ and CUX1$^+$/P73$^+$, Cajal-Retzius precursors were observed in the marginal zone (Abraham et al., 2004; Hodge et al., 2013) (Fig. 1*S*). In this layer, various subpopulations of cells - CUX1$^+$/TBR2$^-$ and CUX1$^+$/P73$^-$ were detected, suggesting that other cell types could be present.

13 gestational weeks

At this stage three cases were analyzed. The anlage of the DG became better delineated compared to previous stage (Fig. 2*A*). For lower magnification see figure 164B from the atlas by Bayer and Altman (Bayer and Altman, 2005).

Enlargement of the secondary dentate matrix

At this stage, PAX6$^+$ cells and proliferating Ki67$^+$ cells appeared less dense in the dentate VZ (Fig. 2*A*, *B-D*) compared to the secondary dentate matrix and anlage (Fig. 2*E-G*). Compared to the previous stage, the percentage of proliferating cells remained relatively stable in the dentate VZ (27±1% of all cells), whereas it increased by about 50% in the dentate anlage. In the secondary dentate matrix and anlage, several Ki67$^+$ cells were co-labeled with PAX6$^+$ (Fig. 2*B*, *E*) or TBR2$^+$ (Fig. 2*D, G*). In particular, in the dentate anlage, PAX6$^+$/Ki67$^+$ and TBR2$^+$/Ki67$^+$ cells represented 29±2% and 28±6 of all PAX6$^+$ and TBR2$^+$ cells, respectively.

Moreover, the presence of colabeled PAX6$^+$/TBR2$^+$ (Fig. 2*C,F*) cells suggested the production of TBR2$^+$ intermediate progenitor cells from PAX6$^+$ progenitors.

In summary, at this stage, the secondary dentate matrix and dentate anlage increased in size, while the proliferative activity of the dentate VZ started to decline.

Postmitotic neurons of the dentate anlage

Postmitotic neurons of the dentate anlage were labeled for CTIP2 (Fig. 2*H, J*) and CUX1 (Fig. 2*I, K*) as in the previous stage. CUX1 labeling was detected in PAX6$^+$ (Fig. 2*K*) and TBR2$^+$ (Fig. 2*I*) cells of the dentate anlage, suggesting that CUX1$^+$ cells might be produced by both PAX6$^+$ and TBR2$^+$ precursors. TBR1 immunoreactivity increased compared to the previous stage displaying positive cells in the fimbrial and dentate VZ, in the secondary dentate matrix and in the dentate anlage (Fig. 2 *L*, *M*).

Fimbrial progenitors and postmitotic neurons

The fimbrial VZ contained several SOX2$^+$ and PAX6$^+$ progenitors (Fig. 3 *A*-*E*) that displayed a proliferation gradient, increasing from the dorsal to the ventral fimbria (Fig. 3*B, C*). PAX6$^+$ cells in the VZ were labeled for CUX1 (Fig. 2*K*). Cycling SOX2$^+$ (Fig. 3*B, C*) and PAX6$^+$ (Fig. 3*D*) progenitors were also observed in the upper ventricular zone, where they formed a tangential band near the superficial part of the fimbria (Fig 3*A*). They were not labeled for either reelin (Fig. 3G) or P73. In the upper ventricular zone, TBR2$^+$/Ki67$^-$ cells were more numerous than TBR2$^+$/Ki67$^+$ cells (Fig. 3*F*), they were associated with CUX1$^+$/P73$^+$, CUX1$^-$/P73$^+$ cells, but also with CUX1$^+$/TBR2$^-$ and CUX1$^+$/P73$^-$ cells (Fig. 3*H, I*). In the superficial part of the fimbria, rare reelin$^+$ cells were detected (Fig. 3*G*), indicating that a few mature Cajal-Retzius cells were present at this stage.

In conclusion, the ventral fimbria contained proliferative progenitors; Cajal-Retzius cell precursors and postmitotic CUX1+/TBR2- or CUX1+/P73- neurons in the superficial part of the upper ventricular zone (Fig 3I) potentially migrating into the dentate anlage.

16-17 gestational weeks

The two cases we analyzed showed that the hippocampal formation had gyrated and the hippocampal fissure had started to close. The DG had enlarged, displaying a thick granule cell layer clearly discernible from the hilus. The external limb of the dentate gyrus (or suprapyramidal blade) was at a more advanced stage of development compared to the internal limb (or infrapyramidal blade), as shown previously (Arnold and Trojanowski, 1996). The dentate VZ was reduced to a thin layer. Within the fimbria, the VZ was still visible (Fig. 4*A*).

Formation of the subpial and hilar matrix

In the thin dentate VZ, cycling $PAX6^+$ progenitors were scarcer than at earlier younger stages, comprising only 6±1% of all $PAX6^+$ cells (Fig. 4*B*). $PAX6^+$ (Fig. 4*C*) and $TBR2^+$ (Fig. 4*F*) progenitor cells appeared denser in the secondary dentate matrix in comparison to the VZ and were mainly found in the subpial stream that extended towards the developing inner limb of the granule cell layer and into the hilus (Fig. 4*B,E*). Some of the $PAX6^+$ (Fig. 4*B,C*) and $TBR2^+$ progenitor cells (Fig. 4*E,F*) displayed proliferative activity in the secondary dentate matrix and in the hilus. In the hilus, proliferating $PAX6^+$ and $TBR2^+$ cells were less dense than at the previous stage, being reduced to 11±2% and 8±4% of all $PAX6^+$ and $TBR2^+$ cells, respectively.

In summary, at this stage, the proliferative activity of the VZ and the deep secondary dentate matrix declined. The main germinal areas consisted of the subpial stream of the secondary dentate matrix extending towards the developing internal limb and into the hilus.

Postmitotic dentate granule cells

In the dentate anlage, CUX1$^+$ and CTIP2$^+$ neurons appeared dense. CTIP2$^+$ neurons were mainly detected in the granule cell layer, whereas CUX1 labeling appeared both in the granule cell layer and in the hilus (Fig. 4*H*). TBR1$^+$ neurons appeared denser compared to previous stages, locating in the hilus and deep granular layer (Fig.4*G*). Some CTIP2$^+$ neurons were double-labeled with TBR1 in the deep granular cell layer and the hilus (Fig. 4*G*). The granule cell layer was composed of numerous CTIP2$^+$/CUX1$^+$ neurons. However, while some CTIP2$^-$/CUX1$^+$ neurons were scattered in the superficial part of the DG, they were more numerous in the hilus (Fig. 4*H*). A few neurons, labeled for NeuN, were observed in the superficial granule cell layer of the external limb (Fig. 4*I*) while deep cells were not labeled, suggesting an outside-in gradient of NeuN expression.

Decrease of progenitor cells in the fimbrial compartment

In the fimbria, PAX6$^+$ cells continued to be observed in the VZ and upper ventricular zone (Fig. 4*J*). However, only a few PAX6$^+$ (Fig. 4*J*) and TBR2$^+$ cells (Fig. 4*K*) still expressed the cycling marker Ki67. Moreover, the presence of a few CUX1$^+$/Ki67$^+$ cells (Fig. 4*L*) suggested that terminal neurogenic divisions might mainly occur in the fimbrial upper ventricular zone.

19-20 gestational weeks

In the two cases we analyzed, both limbs of the granule cell layer were clearly identifiable at posterior levels, whereas the external limb was more developed at anterior levels (Fig. 5*A*), suggesting a caudorostral gradient of development. The fimbria was greatly enlarged and mainly composed of fiber tracts, as cell density was reduced in the upper ventricular zone.

Expansion of the subpial secondary matrix

As observed at GW 16, proliferative PAX6$^+$ progenitors appeared significantly reduced in the dentate VZ but persisted in the subpial area of the secondary dentate matrix (Fig. 5*B*, *C*),

where PAX6$^+$ and TBR2$^+$ progenitor cells were denser (compare Fig. 5*C-E* and *F-H*). Moreover, the presence of PAX6$^+$/TBR2$^+$ cells suggested the continued production of intermediate progenitor cells from PAX6$^+$ progenitors (Fig. 5*G*).

In the hilus, PAX6$^+$ and TBR2$^+$ progenitor were visible (Fig. 5*F-H*), along with PAX6$^+$/Ki67$^+$ cells (Fig. 5*F*). Some PAX6$^+$ and TBR2$^+$ cells were colabeled for NeuroD1, indicating that they were committed to a neuronal lineage (Fig. 5*D*).*Dentate neurons*

Dentate granule cells of the external limb expressed CTIP2 and/or CUX1 (Fig. 5*J,K,L*). CTIP2 showed an increasing staining intensity from deep to superficial cells in the iDG (Fig. 5I) appearing more intense in the eDG (Fig. 5K). CUX1 labeling appeared stronger in the hilus (Fig. 5*J,K*) and in the internal limb of the granule cell layer, still being formed (Fig. 5*I,J*). Rare SATB2$^+$ cells were detected within the hilus where TBR1+ cells were less dense compared to GW 16 (Fig. 5*L*). NeuN$^+$ neurons were found in the superficial granule cell layer of the external limb and in the hilus (Fig. 5*M*).

In summary, an outside-in gradient of neuronal maturation was observed in the granular cell layer, although a clear pattern of layering was not detected.

Fimbrial maturation

PAX6$^+$ precursor cells were still observed in the fimbrial VZ and upper ventricular zone. However, only a few cells displayed proliferating activity (Fig. 5*N*). In addition, only TBR2$^+$/Ki67$^-$ cells remained near the VZ (Fig. 5*O*), suggesting a decline in neurogenic divisions. Some CUX1$^+$ postmitotic neurons were still visible in the upper ventricular zone/superficial part of the fimbria at its junction with the subpial stream oriented towards the DG (Fig. 5*J*). This location suggests that cells of fimbrial origin could contribute to the formation of the granule cell layer.

22-25 gestational weeks

In the three cases we analyzed, both limbs of the DG were formed (Fig. 6*A*).

Dentate matrixes

Ki67 labeling was not observed in the VZ and was reduced in the secondary dentate matrix (Fig. 6*B*), labeling some cells located at the border of the internal limb (Fig. 6*B, C*). In this area, rare $PAX6^+/Ki67^+$ cells (Fig. 6*B*) and some $PAX6^+/NeuroD1^+$ cells (Fig. 6*C*) were observed.

Within the hilus, some $Ki67^+$ cells were still observed (Fig. 6*A, D*). $PAX6^+$ positive cells were homogenously distributed (Fig. 6*D, E*), and a few of them were colabeled for Ki67 (Fig. 6*D*) or NeuroD1 (Fig. 6*E*). $TBR2^+$ cells were not seen in the examined sections (Fig. 6*F*), suggesting reduced neurogenesis.

In summary, the proliferative and neurogenic capability of the two matrixes seemed to decline after mid-gestation.

Postmitotic dentate neurons

In the granule cell layer, CTIP2 immunoreactivity seems to be decreased between GW23 and GW25 (Fig. 6*G, H*), although it still predominated in the superficial granule cell layer. CUX1 labeling was increased in the two limbs compared to previous stages (Fig. 6*H*). Immunoreactivity for BRN2 (Dominguez et al., 2013), another marker of the upper neocortical layers, was not detected in the DG (not shown), indicating a possibly specific role for CUX1 in the specification of the DG.

NeuN labeling still remained restricted to superficial granule cells, suggesting that the maturation of the granule cell layer was not complete at this stage (Fig. 6*I*).

Fimbria

Within the fimbria, $PAX6^+$, but not $TBR2^+$, cells could be observed. $PAX6^+/Ki67^+$ cells were rare, confirming the reduction in proliferative activity that began during the previous stages (Fig. 6*J*). The density of $CUX1^+$ cells was reduced compared to GW20, but they were still detected at the junction with the subpial stream (Fig. 6*K*).

Evolutionary divergence in the expression pattern of cell-fate markers

To assess whether differences could exist between the human and mouse, we extended the characterization of postmitotic markers to the DG of mice, using the same antibodies for all markers except Cux1. The granule cell layer of postnatal mice at P5 and P10 displayed increasing Ctip2 labeling from deep to superficial neurons, similar to that observed in human fetuses between GW16 and GW20. Moreover, Satb2 was predominantly detected in the hilus and rarely observed in granule cells in mice, as observed in our human samples. Conversely, Cux1 staining was not detected in the mouse granule cell layer - a major discrepancy with respect to human data; however the same antibody could not be used. Overall these results may indicate different molecular pathways involved in the specification of granule cells in the two species.

Discussion

In this paper we investigated the different steps in the formation of dentate germinal layers and postmitotic neurons from GW9 to GW25. We also characterized progenitors and postmitotic neurons in the fimbrial anlage.

Proliferating areas of the developing human DG

Our study shows, using Ki67 labeling, that even at very early stages (GW11), the putative dentate VZ is distinguishable from the adjacent ammonic VZ (Cipriani et al., 2015). The

secondary dentate matrix appears between GW10 and GW11 within the intermediate zone, when the anlage of the DG becomes recognizable. This indicates that, as shown in rodents, the two matrices participate in the generation of the first granule cells (Altman and Bayer, 1990b). From GW13 onward, the secondary dentate matrix becomes the main germinal area of the DG. Then, between GW16 and GW20, the secondary dentate matrix is reduced around the external limb, while it forms the hilar and subpial germinal compartments, adjacent to the nascent granule cell layer, near the less developed internal limb. This sequential formation of proliferating areas in the developing DG is in agreement with previous findings in rodents (Altman and Bayer, 1990a, b; Nakahira and Yuasa, 2005; Hodge et al., 2012). Between GW22 and GW25, when the internal limb of the granule cell layer becomes recognizable, the secondary dentate matrix is also reduced in the subpial area, while some progenitor cells remain in the hilus. This is consistent with the formation of a subgranular zone at later stages that generates neurons during adult human life (Eriksson et al., 1998; Knoth et al., 2010), as previously described in rodents (Altman and Bayer, 1990a; Hodge et al., 2012; Hodge et al., 2013).

We have also shown the presence of cycling $PAX6^+$ and $TBR2^+$ progenitor cells within the secondary dentate matrix and in the hilus from GW9-11, and lasting until GW20. These findings indicate that neurogenesis starts at early stages of hippocampal development in parallel with the development of the pyramidal layer (Cipriani et al., 2015), and are in favor of a role for TBR2 in human granule cell neurogenesis. Tbr2 expression has been reported to be required for neuronal lineage progression to granule cells in newborn mice (Hodge et al., 2012; Hodge et al., 2013; Roybon et al., 2009). In our samples, the presence of $PAX6^+/TBR2^+$, $TBR2^+/NeuroD1^+$ and $TBR2^+/CTIP2^+$ cells suggest that TBR2 function is conserved in neuronal lineage progression in the human developing hippocampus. Moreover, CUX1 labeling in both $TBR2^+$ and $PAX6^+$ cells suggests that $CUX1^+$ neurons might be derived from both $TBR2^+$ and $PAX6^+$ precursors. Further characterization of the DG

development and the role of TBR2 and PAX6 might elucidate whether evolutionary differences exist between primates and rodents.

What is the role of the fimbria in the development of the human dentate gyrus?

The hem VZ has been shown to be a source of hippocampal Cajal-Retzius cells in humans and mice (Abraham et al., 2004; Gu et al., 2011; Hodge et al., 2013). The proliferative activity and subtypes of neuronal progenitor cells have been partly studied in the hem/fimbria, with a focus on the formation of Cajal-Retzius cells (Abraham et al., 2004). Our data show that proliferating PAX6 and SOX2 progenitors are located in the hem VZ and subpial layer at early stages (GW10-11), and continue to be detectable until GW25. In fact, the density of tangentially-oriented $SOX2^+$ and $PAX6^+$ cells increases towards the dentate anlage between GW11 and GW13. These data suggest that the hem/fimbria might also be a source of progenitor cells for the DG. Interestingly, Altman and Bayer have reported that the secondary dentate matrix contains a "component of extra-dentate origin" in rats (Altman and Bayer, 1990a).

In humans, Cajal-Retzius cells have been shown to be generated at the fimbrio-dentate boundary after the regression of the hem (GW13) (Abraham et al., 2004). However the fate of the remaining fimbrial progenitors is not known. In mice, Cajal-Retzius cells have been shown to express Tbr2 (Gu et al., 2011; Hodge et al., 2013), which is required for their correct migration (Hodge et al., 2013). Similarly, we have observed $TBR2^+/Ki67^-$ cells in the fimbrial upper ventricular zone until GW13. These may correspond to Cajal-Retzius cells, which have recently been shown to express CUX1 (Cubelos et al., 2008). However, in this area, other cell populations, consisting of $CUX1^+/TBR2^-$ and $CUX1^+/P73^-$ cells, have been observed from early stages until GW25. Further characterization of these cells in mice and humans is thus necessary.

Granular cell layer

Dentate granule cells are morphologically homogenous in all species (Scharfman, 2011), however, the mechanisms regulating the formation and the maturation of DG in two limbs are not fully understood. We have demonstrated the labeling of young neurons in the dentate anlage with post-mitotic markers as early as GW11, before the formation of the granule cell layer. When the external limb of the granule cell layer become identifiable, around GW16 (Arnold and Trojanowski, 1996), CTIP2 and TBR1 labeling was observed in granular and hilar cells, respectively, suggesting different roles for the two transcription factors in the development of the dentate formation. Although the role of cell fate markers has not been deeply investigated in the dentate gyrus, experimental studies have shown that CTIP2 is required for correct granule cell differentiation. CTIP2 would exert feedback control on dentate progenitor cells stimulating their proliferation (Simon et al., 2012). In support of a similar function in human granule cell generation, we showed that CTIP2 expression is attenuated after GW23, when the two limbs of the DG are formed and the progenitor cell pool is reduced. Moreover, in mice, CTIP2 ablation causes aberrant mossy fiber projections to the CA3 field, associated with a deficit in learning and memory (Simon et al., 2012). In humans, at 16 weeks, the superficial layer of granule cells starts to express NeuN, in parallel with the CA3 field (Cipriani et al., 2015). This may represent the first step in mossy fiber projection, which in humans, similar to rats, mostly occurs during the postnatal period (Seress and Mrzljak, 1992). However, in humans, we have reported that CTIP2 labeling is reduced to almost nothing after mid-gestation, whereas it continues to be detected in mice during the postnatal period. This suggests that in humans, other molecules might regulate mossy fiber projection at later fetal stages and during the postnatal period.

Granule cells are also labeled for CUX1 (Cubelos et al., 2010; Li et al., 2010) until GW25. Cux1 is known to regulate dendrite and synapse formation in pyramidal cells, for the establishment of cortico-cortical connections (Cubelos et al., 2010; Li et al., 2010). In mammals, the axons of dentate granule cells (hippocampal mossy fibers) project not only to

the hippocampal CA3 field but also to the dentate hilus (the polymorphic layer or CA4) (Insausti and Amaral, 2012). Thus, the differential expression of CTIP2 and CUX1 might play a role in regulating the formation of different types of dentate projections.

Although we did not observe a typical layering pattern within the granule cell layer, as we did in the case of the ammonic plate (Cipriani et al., 2015), from GW16, NeuN labeling displays an outside-in gradient of expression that becomes more evident at later stages while still predominating in superficial layers. This represents a maturation gradient that is inverted with respect to the gradients reported in the neocortex and in the pyramidal layer of the hippocampal formation (Sidman and Rakic, 1973; Kostovic et al., 1989; Arnold and Trojanowski, 1996; Sarnat et al., 1998; Bayatti et al., 2008; Abraham et al., 2009; Saito et al., 2010; Cipriani et al., 2015) and this is in agreement with previous experimental studies that have reported an outside-in gradient of neurogenesis within the granule cell layer (Altman and Bayer 1990).

Evolutionary comparison of cell-fate-specific transcription factors

We have shown that the murine expression pattern of Ctip2 and Satb2 is conserved in the human DG. The role of Ctip2 in murine granule cells has begun to be experimentally elucidated, as discussed above (Simon et al., 2012). Satb2 represses Ctip2 in the neocortex, but its function in the hippocampus is not known. Satb2 mutants have a severely reduced corpus callosum, whereas the anterior and hippocampal commissures remain intact (Alcamo et al., 2008).

Altogether, these findings suggest that, as in the neocortex (Alcamo et al., 2008; Chen et al., 2008), layer markers might play a role in determining dentate connections, and that some of these factors might have been conserved during evolution. However, differences have also been found. In rodents Tbr1 has been reported to be highly expressed in maturing dentate granular cells (Roybon et al., 2009; Hodge et al., 2012). In human samples, TBR1 labeling was mainly observed in hilar cells compared to granule cells. Thus, TBR1 would be less

expressed in human compared to rodent dentate cells (Roybon et al., 2009; Hodge et al., 2012) and also compared to human hippocampal pyramidal cells (Cipriani et al., 2015). Its role in the dentate gyrus has not been completely elucidated yet. TBR1 might either play a selective role in hilar neurons maturation or be downregulated in newly generated granular cells as soon as they rich the deep granular cell layer.

Conversely, lack of Cux1 immunoreactivity was found in the mouse dentate gyrus, whereas it was extensively detected in human fetuses. In the neocortex, CUX1 regulates the formation of cortico-cortical connections (Cubelos et al., 2010; Li et al., 2010). Although we cannot exclude that such a difference is due to the immunosensitivity of the antibodies we used, massive CUX1 expression might reflect differences in connectivity between species. One example is the commissural connection. While cells of the polymorphic layer give rise to a very robust commissural projection to the molecular layer of the contralateral dentate gyrus in the rat, this projection does not exist in the non-human primate brain or in the human brain (Amaral et al., 2007). Overall, these observations suggest that further studies in human and primate would be necessary to investigate the role of these transcription factors in the development and connectivity of the dentate gyrus.

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Legends to figures

Figure 1. The human cortical hem and dentate anlage at GW9 (*A-E*) and GW11 (*F-S*). Single and double (yellow) immunofluorescent labeling. (*A*) Nissl staning of the human brain at GW9. (*B*) Nissl staining showing the ventral hem. (*C*) DAPI staining of the ventral hem and the primordial hippocampus. The limits between the two structures cannot be determined yet. The VZ, intermediate zone and the marginal zone are recognizable. Asterisk marks the area where confocal pictures D and E were taken. (*D*) Proliferating $SOX2^+$ and (*E*) $PAX6^+$ progenitors of the hem/dVZ. The asterisk in E indicates the putative secondary dentate matrix. (*F*) $Ki67^+$ cells in the hippocampal formation. Arrows indicate streams of $Ki67^+$ cells within the hem and around the dentate anlage. (*G*) Proliferating $PAX6^+$ progenitors of the dentate VZ and secondary dentate matrix (ds). (*H*) $PAX6^+/TBR2^+$ cells are observed in the ds. (*I*) $TBR2^+$ cells are present in the VZ and SVZ. Proliferating $Ki67^+/TBR2^+$ progenitors (arrows) are visible in the secondary dentate matrix. (*J*) $PAX6^+/TBR2^+$ cells extend towards the dentate anlage. (*K*) Proliferating $TBR2^+$ intermediate progenitor cells (yellow) are visible around the dentate anlage. (*L*) NeuroD1 staining intensity increases towards the subpial layer in $TBR2^+$ cells surrounding the DA. Cells in the dentate anlage are labeled for NeuroD1. (*M*) CTIP2/CUX1 double-labeling within the dentate anlage. CTIP2/CUX1 double-labeled cells predominate among the peripheral cells of the dentate anlage, surrounding $CTIP2^+$ cells. (*N*) TBR1 labeling is very faint in the dentate area, while (O) positive cells are detected in the adjacent ammonic plate. (*P-R*) $TBR2^+$cells of the hem. (*P*) $TBR2^+/PAX6^+$ (yellow) and $TBR2^+/PAX6^-$ cells (arrows) are abundant in the superficial part of the intermediate zone. (*Q*) $TBR2^+$ cells do not show detectable proliferative activity. (*R*) Numerous CUX1-expressing cells are present in the VZ and intermediate zone, with a few coexpressing $TBR2^+$. (*S*) $P73^+/CUX1^-$ cells are present in the hem. Dashed line in F, J and K delimitates the DA. Scale bar: 600 µm in A, 200 µm in B, C and F, 50 µm in D, E, and G-S. aVZ, ammonic ventricular zone; DA, dentate anlage; ds, secondary dentate matrix; d, dorsal; dVZ, dentate ventricular zone; GE, ganglionic eminence; IZ, intermediate zone; MZ, marginal zone; l, lateral; m,

medial; v, ventral; VZ, ventricular zone.

Figure 2. Analysis of progenitors and cell-fate-specific transcription factors in the dentate area at GW13. (*A*) Ki67 labeling and counterstaining with hemalum. Arrows indicate the secondary dentate matrix stream from the dentate notch. (*B*) Cycling $PAX6^+$ progenitors (arrows) of the dentate VZ and secondary dentate matrix, (*C*) $PAX6^+/TBR2^+$ cells (arrows) are observed in the secondary dentate matrix. (*D*) Cycling $TBR2^+$ progenitors (arrows) are visible in the secondary dentate matrix. Asterisks in *C* and *D* indicate the limit between the putative dentate (left) and ammonic VZ (right). (*E*) Cycling PAX6+ progenitors (arrows) of the secondary dentate matrix extend towards the dentate anlage. (*F*) $PAX6^+/TBR2^+$ cells (arrows) are observed in the secondary dentate matrix and in the dentate anlage. (*G*) Cycling $TBR2^+$ progenitors (yellow) of the secondary dentate matrix extend towards the dentate anlage. (*H*) In the dentate anlage, $PAX6^+$ cells and $CTIP2^+$ neurons are present. (*I*) CUX1 and (*J*) CTIP2 are detected in $TBR2^+$ cells (arrows). (*K*) CUX1 is expressed in $PAX6^+$ cells of the secondary dentate matrix (arrowheads) and fimbrial VZ (arrows). (*L*) Rare $TBR1^+$ cells are visible in the fimbria-dentate area, (*M*) where they are co-labeled with CUX1. Scale bar: 200 µm in A, 50 µm in B-J, L and M, 25 µm in K. d, dorsal; DA, dentate anlage; ds, secondary dentate matrix; dVZ, dentate ventricular zone; FI, fimbria; l, lateral; m, medial; v, ventral; VZ, ventricular zone.

Figure 3. Analysis of progenitors and cell-fate-specific transcription factors in the fimbria at GW13. (*A*) Tiled image of a coronal section showing the distribution of $SOX2^+$ cells from the dorsal to the ventral dentate anlage (see also Supplementary Fig.1 for DAPI and Nissl stainings). B and C indicate the areas used for confocal imaging. A $SOX2^+$ cell band is visible in the upper ventricular zone of the fimbria (arrows). (*B, C*) Proliferating $SOX2^+$ cells (arrows) are observed in the VZ and upper ventricular zone of the ventral fimbria. (*C*) Rare proliferating cells are detected in the VZ close to the junction with the dorsal fimbria. (*D*) Proliferating $PAX6^+$ cells are observed in the VZ and upper ventricular zone of the ventral fimbria (arrows). (*E*) PAX6/vimentin colabeling showing the tangential orientation of

progenitor cells within the upper ventricular zone. (*F*) TBR2$^+$ cells of the fimbria are rarely labeled for Ki67. Double-labeled cells (arrows) are observed near the dentate anlage. (*G*) A few reelin$^+$ cells are observed in the fimbria. (*H*) Few CUX1$^+$ cells express TBR2 or (*I*) P73. In I arrows indicate the cell stream from the fimbria to the DA. In *I,* arrows indicate CUX1+/P73- cells in the superficial part of the upper ventricular zone. Scale bar: 500 µm in A, 50 µm in B-I. d, dorsal; DA, dentate anlage; dVZ, dentate VZ; FI, fimbria; fVZ, fimbrial VZ; l, lateral; m, medial; v, ventral.

Figure 4. Analysis of progenitors and cell-fate-specific transcription factors in the dentate area at GW16. (*A*) Nissl staining. Asterisk indicates the subpial compartment of the secondary dentate matrix. Dashed line indicates the hippocampal fissure. *b-l* indicate the area of confocal pictures *B-L*. (*B*) A few cycling PAX6$^+$ cells (yellow) are observed in the dentate VZ. They were numerous in the secondary dentate matrix, increasing in density toward the granule cell layer (displayed in yellow in *C*) and in the dentate hilus (arrows in *D*). (*E*) Rare cycling TBR2+ cells (arrows) are observed in the dentate VZ and in the adjacent secondary dentate matrix. (*F*) Cycling TBR2$^+$ cells (yellow) in the subpial matrix increase in density toward the internal limb of the granule cell layer. (G) TBR1$^+$ cells are observed in the hilus and deep granular layer. (*H*) CTIP2$^+$ and CUX1$^+$ cells of the dentate gyrus. CTIP2+ and/or CUX1$^+$ cells (yellow) are visible within the granule cell layer. (*I*) NeuN$^+$ neurons are detected in the superficial granule cell layer and hilus. (*J*) Cycling PAX6$^+$ and (*K*) TBR2$^+$ cells (arrows) are visible in the fimbria. (L) Some cycling cells express CUX1 in the fimbria. Scale bar: 500 µm in A, 50 µm in B-G and I-K, 25 µm in H. ds, secondary dentate matrix; ds, secondary dentate matrix; d, dorsal; dVZ, dentate ventricular zone; eDG, external limb; fVZ, fimbrial ventricular zone; GL, granule cell layer; l, lateral; m, medial; sp, subpial stream; v, ventral.

Figure 5. Analysis of progenitors and cell-fate-specific transcription factors in the dentate area at GW20. (*A*) Nissl staining. (*B*) Distribution of Ki67$^+$ cells within the dentate formation. In A and B arrows indicate the secondary dentate matrix stream. (*C*) PAX6$^+$/Ki67$^+$ cells

(arrows) are located in the secondary dentate matrix but not in the dentate VZ. (*D*) PAX6$^+$/NeuroD1$^+$ (ND1) cells (yellow) are observed in the dentate VZ and secondary dentate matrix. (*E*) Few TBR2$^+$/NeuroD1$^+$ (ND1) cells are detected in the secondary dentate matrix. (*F*) PAX6$^+$/Ki67$^+$, (*G*) PAX6$^+$/TBR2$^+$ and (*H*) TBR2$^+$/Ki67$^+$ colabeled cells (yellow) reflect the presence of proliferating progenitors extending from the secondary dentate matrix towards the dentate hilus. (*I*) PAX6/CTIP2/CUX1 colabeling showing the limit between the secondary dentate matrix and the internal limb of the granule cell layer. CTIP2$^+$ cells of the internal limb display strong CUX1 labeling (yellow), PAX6$^+$ and/or CUX1$^+$ cells are present in the hilus and in the subpial stream. (*J*) CUX1$^+$ cells are present in the hilus, in the secondary dentate matrix and in the subpial stream (arrows). Inset: higher magnification picture of the subpial stream. (*K*) CTIP2/CUX1 colabeling showing single and double-labeled cells in the thicker part of the granule cell layer, whereas CUX1$^+$/CTIP2$^-$ neurons predominate in the hilus. (*L*) TBR1/SATB2/DAPI colabeling showing rare TBR1$^+$ and SATB2$^+$ cells in the dentate hilus. (*M*) NeuN$^+$ cells are detected in the superficial granule cell layer and hilus. Dashed line indicates the limit between the granule cell layer and hilus. (*N*) Cycling PAX6$^+$ progenitors are visible in the fimbrial upper ventricular zone and VZ. Arrowheads indicate the stream of the secondary dentate matrix. Asterisk indicates the localization of the VZ. (*O*) Rare TBR2$^+$/Ki67$^-$ cells are still detected near the fimbrial VZ (arrows). Scale bar: 500 µm in A, 200 µm in B and in O, 50 µm in C-E, I-J, L and N, 25 µm in F-H, K and M. DG, dentate gyrus; ds, secondary dentate matrix. d, dorsal; dVZ, dentate ventricular zone; eDG, external limb; iDG internal limb; FI, fimbria; l, lateral; m, medial; v, ventral; VZ, ventricular zone.

Figure 6. Analysis of progenitors and cell-fate-specific transcription factors in the dentate area at GW23-25. (*A*) Localization of Ki67$^+$ cells within the dentate formation. (*B*) Cycling PAX6$^+$ cells (arrows) and (*C*) PAX6$^+$/NeuroD1$^+$ (yellow) cells are detected in the remaining secondary dentate matrix at the border of the internal limb. Note that some cells display cytoplasmic PAX6 labeling. Few (*D*) PAX6$^+$/Ki67$^+$ cells and (*E*) PAX6$^+$/NeuronD1$^+$ cells (arrows) are detected in the dentate hilus. (*F*) TBR2$^+$ cells are not detected within the dentate

gyrus. (*G*) CTIP2^{+} and CUX1^{+} cell distribution in the granule cell layer at GW23 and (*H*) GW25. Note the reduced CTIP2 labeling at GW25. (*I*) NeuN^{+} cells are observed in the superficial granule cell layer and hilus. (*J*) Rare PAX6^{+}/Ki67^{+} cells are visible in the fimbria among fibers. (*K*) CUX1^{+} cells form a stream from the fimbria towards the dentate gyrus (arrows). Scale bar: 200 µm in A, 50 µm in B-E and G-J, 25 µm in F, 500 µm in K. d, dorsal; DG, dentate gyrus; eDG, external limb; FI, fimbria; iDG internal limb; l, lateral; m, medial; v, ventral.

Figure 7. Analysis of cell-fate-specific transcription factors in the dentate gyrus of newborn mice. (*A*) Relative localization of Ctip2, Satb2 and Cux1 in granule cells at P5 and (*B*) P10. Note that Cux1 is not detected in the dentate area. Scale bar: 200 µm. c, caudal; d, dorsal; DG, dentate gyrus; l, lateral; m, medial; r, rostral; v, ventral.

Table1

Human fetuses employed in the study

Cases	Age in gestational weeks	Tissue processing	Postmortem delay/fixation time
1	9	paraffin	NA
2	9	paraffin	NA
3	10	paraffin	NA
4	11	frozen	2h/24h
5	11	frozen	2h72h
6	13	frozen	15h/15h
7	13	frozen	15h/15h
8	13	frozen	NA
9	16	frozen	24h/48h
10	17	paraffin	NA
11	19	frozen	24h/72h
12	20	frozen	10h/10h
13	22	frozen	8h/24h
14	23	paraffin	NA
15	25	frozen	NA

Table 2

Antibodies employed in the study

Antibody	Company	Species	Concentration used on frozen/paraffin sections	Target	References
Proliferation					
Ki67	Abcam, ab27619	Rabbit	1:200/-	Cell cycle related nuclear protein	(Bento et al., 2010)
Ki67	Dako, M7240 (Clone MIB)	Mouse	1:500/1:50	Cell cycle related nuclear protein	(Gerdes et al., 1983)
Neuronal progenitors					
SOX2	Abcam, ab97959	Rabbit	1:200/1:100	Stem cell self-renewal transcription factor	(Lundberg et al., 2014)
PAX6	Proteintech, 12323-1-AP	Rabbit	1:100/1:100	Stem cell trascription factor	(Kreitzer et al., 2013)
PAX6	DSHB	Mouse	1:10/-	Stem cell trascription factor	(Ericson et al., 1997)
TBR2	Abcam, ab23345	Rabbit	1:200/1:50	Transcription factor present in neurogenic intermediate progenitors	(Fietz et al., 2010)
Vimentin	Leica, VIM-572-L-CE	Mouse	1:500/-	Intermediate filament protein	(Teissier et al., 2014)
Post-mitotic cells					
TBR1	Abcam, ab31940	Rabbit	1:500/-	Transcription factor regulating the maturation of post-mitotic cells	(Bayatti et al., 2008)
CTIP2	Abcam, ab18465	Rat	1:500/1:200	Transcription factor regulating the maturation of post-mitotic cells	(Johnson et al., 2009)
SATB2	Abcam, ab18465	Mouse	1:200/-	Transcription factor regulating the maturation of post-mitotic cells	(Ip et al., 2011)
CUX1	Abcam, ab54583	Mouse	1:500/1:500	Transcription factor regulating the maturation of post-mitotic cells	(Hadjivassiliou et al., 2010)
NeuroD1	Abcam, ab60704	Mouse	1:500/-	Neuronal differrentiation promoting transcription factor	(Roybon et al., 2009)
P73	Abcam, ab14430	Rabbit	1/10,000*/-	Tumor suppressor protein	(Cabrera-Socorro et al., 2006)
Reelin	Gift of Pierani A.	Mouse	1/500/-	Extracellular matrix glycoprotein	(Bielle et al., 2005)
NeuN	Millipore, MAB377	Mouse	1:500/-	Neuron-specific nuclear protein	(Jager et al., 2013)

*Immunoreactivity was visualized by Tyramide *Signal* Amplification System (PerkinElmer, Waltham, MA)